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Cloning and Functional Studies of a Novel Gene Aberrantly Expressed in RB-Deficient Embryos

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The tumor suppressor RB regulates diverse cellular processes such as G1/S transition, cell differentiation, and cell survival. Indeed, *Rb*-knockout mice exhibit phenotypes including ectopic mitosis, defective differentiation, and extensive apoptosis in the neurons. Using differential display, a novel gene, *Rig-1,* was isolated based on its elevated expression in the hindbrain and spinal cord of *Rb*-knockout embryos. The longest open reading frame of *Rig-1* encoded a polypeptide that consists of a putative extracellular segment with five immunoglobulin-like domains and three fibronectin III-like domains, a putative transmembrane domain, and a distinct intracellular segment. The Rig-1 sequence was 40% identical to the recently identified roundabout protein. Consistent with the predicted transmembrane nature of the protein, Rig-1 protein was present in the membranous fraction. Antisera raised against the putative extracellular and intracellular segments of Rig-1 reacted with an ~210-kDa protein in mouse embryonic CNS. Rig-1 mRNA was transiently expressed in the embryonic hindbrain and spinal cord. Elevated levels of Rig-1 mRNA and protein were found in $Rb^{-/-}$ embryos. Ectopic expression of a transmembrane form of Rig-1, but not the secreted form, promoted neuronal cell entrance to S phase and repressed the expression of a marker of differentiated neuron, Tα1 tubulin. Thus *Rig-1,* a possible distant relative of *roundabout,* may mediate some of the pleiotropic roles of RB in the developing neurons.

Key Words: **RB; neuronal cell cycle control; neuronal differentiation; transcriptional regulation.**

INTRODUCTION

The loss of a functional retinoblastoma susceptibility gene, *Rb*, either through the inheritance of a mutant gene followed by a somatic mutation of the other allele or by two consecutive somatic mutations, appears to be a necessary step in human retinoblastoma formation. The *Rb* gene, which encodes a ubiquitously expressed nuclear phosphoprotein, is frequently mutated during cancer progression in other tumors as well. RB plays an important role in regulating G1/S progression and thus may be broadly involved in tumorigenesis (for reviews, see Riley *et al.,* 1994; Sherr and Roberts, 1995; Weinberg, 1995; Skapek *et al.,* 1997).

RB regulates G1/S progression primarily through its interaction with specific members (E2F1–E2F3) of the E2F transcription factor family. E2F family members (E2F1–E2F5) exist as heterodimers with either DP1 or DP2, collectively referred to as E2F (reviewed by Nevins *et al.,* 1997; Dyson, 1998). E2F, a transcriptional activator of several S-phase-promoting genes, is converted into a transcriptional repressor upon binding RB, which in turn results in G1/S block (Weintraub *et al.,* 1992; reviewed by Sellers and Kaelin, 1996). Such a mechanism appears to be evolutionarily conserved since RB, E2F, and DP homologs have been identified even in invertebrates such as *Drosophila* (Du *et al.,* 1996), and dE2F/dDP complex is required for its zygotic proliferation (Dynlacht *et al.,* 1994; Ohtani and Nevins, 1994; Duronio *et al.,* 1995).

In view of the ubiquitous expression of RB and its critical role in cell cycle regulation, deletion of *Rb* in mice revealed a surprisingly restricted phenotype. Heterozygous $Rb^{+/-}$ mice are predisposed to pituitary tumor of the intermediate lobe at nearly 100% penetrance (Jacks *et al.,* 1992; Lee *et al.,* 1992; Hu *et al.,* 1994; Nikitin and Lee, 1997). $Rb^{-/-}$ mouse embryos die before embryonic day 16.0 with multiple defects, including significant abnormalities in the nervous

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system (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Neurons fail to exit the cell cycle even after migrating to the intermediate and migration zones, areas that normally contain postmitotic cells. Deregulation of the neuronal cell cycle is accompanied by a lack of terminal differentiation and apoptosis (Lee *et al.*, 1994; Nikitin and Lee, 1996). $Rb^{-/-}$ mouse embryos show very low levels of βII tubulin expression, a marker for terminally differentiated neurons, and neuronal cells established from their sensory ganglia differentiate poorly and have poor survival rates (Lee *et al.*, 1994). Additionally, embryos that express low levels of wild-type RB protein and survive until birth show specific skeletal muscle defects, including increased cell death prior to myoblast fusion, implicating a role for RB in skeletal muscle development and cell survival (Zacksenhaus *et al.*, 1996). Therefore, the effect of RB inactivation is cell-type specific *in vivo*. Interestingly, these phenomena are not seen in all cell types. For example, proliferation and differentiation of T and B lymphocytes appear to be normal in the absence of RB protein (Chen *et al.*, 1993; Hu *et al.*, 1997).

The cell-type-specific function of RB may be in part due to its interaction with certain cell-specific transcription factors. Indeed RB interacts with specific transcription factors (e.g., ATF-2, MyoD, C/EBP, NF-IL6) and thereby regulates differentiation of myocytes, macrophages, and adipocytes (Chen *et al.*, 1996b; reviewed by Dynlacht, 1997). Monocyte/macrophage differentiation requires the NF-IL6 transcription factor (Natsuka *et al.*, 1992), which is activated upon binding to unphosphorylated RB (Chen *et al.*, 1996a). RB, through its interaction with a muscle-specific bHLH transcription factor, MyoD, induces differentiation of muscle cells and retains them in the differentiated state (Caruso *et al.*, 1993; Gu *et al.*, 1993). Interaction between RB and C/EBP is required for adipocyte differentiation (Chen *et al.*, 1996b). Cell-type-specific phenotypes seen in $Rb^{-/-}$ embryos may also be explained at least partially due to the expression of two additional members of the RB family, p107 and p130, which also interact with E2F and regulate cell cycle progression. Although the level of expression of p107 and p130 in $Rb^{-/-}$ embryos is similar to that in $Rb^{+/+}$ embryos (Chen *et al.*, 1996; Jiang *et al.*, 1997), p107 and RB are differentially expressed during embryogenesis (Jiang *et al.*, 1997). Additionally, the level of p107 is elevated in $p130^{-/-}$ T lymphocytes, suggesting a functional compensation between RB family members (Mulligan *et al.*, 1998).

Based on the observations that RB interacts with tissue-specific transcription factors and that $Rb^{-/-}$ mouse embryos show severe neuronal defects, we hypothesized that RB interacts with certain neuron-specific or neuron-enriched transcription factors, which in turn may regulate neuron-specific gene expression. Here, we report the identification of a novel gene, *Rig-1*, whose expression is aberrant in the $Rb^{-/-}$ mouse embryos and which may be responsible for some phenotypes seen in the nervous system of the $Rb^{-/-}$ mouse embryos.

MATERIALS AND METHODS

Animals

Timed pregnancy was arranged to obtain embryos at the desired stages of gestation. Both wild-type and $Rb^{-/-}$ mouse embryos were obtained from mating *Rb* heterozygous mice (Lee *et al.*, 1992). The mice used were an outbred strain from C57BL/6 and 129/SvEv mice.

mRNA Differential Display

mRNA differential display was performed using mRNAs extracted from hindbrain, spinal cord, and dorsal root ganglia of wild-type and $Rb^{-/-}$ mouse embryos (Liang and Pardee, 1992). The experimental procedure was according to the manufacturer's manual of the RNAmap mRNA differential display system (GenHunter, Brookline, MA).

In Situ Hybridization

Mouse embryos at different gestational stages were fixed in freshly prepared paraformaldehyde solution (4% in PBS, pH 7.0) at 4°C overnight. Paraffin sections were prepared following successive dehydration and embedding of embryos in paraffin. Antisense oligonucleotide probe (5′-ACTGTTGTAGACAGTGTGCTTCAGTGAGTCACAAGCATCATTGCTGGGAG-3′), sequence from the 3′ untranslated region of Rig-1, was end-labeled with [^{33}P]dATP using terminal deoxynucleotidyl transferase (Promega, Madison, WI). Hybridization was carried out at 42°C overnight, followed by washing in 0.1× SSC at 42°C (Cox *et al.*, 1984; Lee *et al.*, 1994). Sense oligonucleotide (5′-TGACAACATCTGTCACACGAAGTCACTCAGTGTTCGTAGTAACGACCCTC-3′) was used as the negative control.

Northern Hybridization

mRNAs were extracted from whole embryos at various stages. Three micrograms of mRNA was mixed with glyoxal/DMSO buffer and loaded onto ATA-containing 1% agarose gel and transferred to Hybond-N membrane (Amersham, Arlington Heights, IL). A 1.4-kb *Xho*I fragment encompassing the 3′ end of the protein-encoding region of Rig-1 was labeled with [^{32}P]dCTP by the random priming method (Stratagene, La Jolla, CA) and used as the probe. After hybridization and wash, the blot was exposed to Kodak XAR5 film and developed after a 1-week exposure (Sambrook *et al.*, 1989).

cDNA Library Screening and DNA Sequencing

A λgt11 cDNA library from 11.5-day-old (E11.5) mouse embryos was used to obtain Rig-1 cDNAs (Clontech, Palo Alto, CA). The screening procedure followed was according to published methods (Sambrook *et al.*, 1989). DNA was sequenced using Sequenase 2.0 (USB, Cleveland, OH) by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977).

Western Blotting Analysis

Total cell lysates were extracted from mouse embryonic tissues and cultured cells using lysis buffer containing 50 mM Tris–HCl (pH 8.0), 120 mM NaCl, 0.5% NP-40, 10 μg/ml aprotenin, 50 μg/ml

leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Rabbit and mouse polyclonal antibodies, raised against amino acids 376–929 (anti-ECD) and amino acids 1067–1234 (anti-ICD) of Rig-1 protein, respectively, were used for immunoprecipitation and Western blot analysis.

Subcellular Fractionation

Cells from hindbrain and spinal cord of E11.5 mouse embryos and Neuro-2A mouse neuroblastoma cells (ATCC, Rockville, MD) transfected with pCEP4F-Rig were subjected to subcellular fractionation (Lee *et al.*, 1987). The pCEP4F-Rig-1 encoded a Flag-tagged Rig-1 (amino acids 1–1344) which was constructed by fusing Rig-1 cDNA in frame into the pCEP4 (Invitrogen, Carlsbad, CA) vector that was modified to encode the Flag tag. For cell fractionation, the cells were harvested and resuspended in the buffer containing 10 mM Hepes (pH 6.2), 10 mM NaCl, 1.5 mM $MgCl_2$, 10 μg/ml aprotenin, 50 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride. After homogenization with a Dounce homogenizer, subcellular fractions were collected as described (Lee *et al.*, 1987). For detecting the subcellular localization of endogenous Rig-1 protein and Flag-tagged Rig-1, rabbit antiserum against the extracellular domain of Rig-1 (amino acids 376–929) and anti-Flag M2 monoclonal antibody (Kodak, Rochester, NY) were used in Western blotting analysis. Monoclonal antibody against p48, which was previously reported (Qian and Lee, 1995), glutathione Sepharose 4B beads (Pharmacia, Uppsala, Sweden), and monoclonal antibody against the deleted in colorectal cancer protein (DCC) (Oncogene Research, Cambridge, MA) were used for detection of p48, GST, and DCC, respectively.

BrdU Labeling and Immunofluorescent Staining

Neuro-2A cells were seeded at a density of 5×10^4/35-mm plate and grown overnight prior to transfection. Rig-1 expression constructs (Rig-TM or Rig-Sec) and a nuclear localization signal-containing β-galactosidase (CS+ nucβ-gal) expression construct (Fire *et al.*, 1990) were cotransfected (5:1 ratio) into Neuro-2A cells. E2F and RB expression constructs as well as their parental vectors were included as controls (Shan *et al.*, 1994; Qin *et al.*, 1992). Twenty-four hours after transfection, BrdU (Sigma, St. Louis, MO) was added to a final concentration of 10 μM and the cells were labeled for 10 h. Cells were fixed with 2% paraformaldehyde. Mouse anti-BrdU monoclonal antibody (1:500) (Becton–Dickinson, San Jose, CA) and rabbit anti-β-galactosidase antibody (1:500) (5 Prime → 3 Prime, Boulder, CO) were added after blocking with 5% milk in TBST. Goat rhodamine-conjugated anti-mouse IgG (Cappel, Durham, NC) and goat FITC-conjugated anti-rabbit IgG (Fisher, Burr Ridge, IL) were used as secondary antibodies and DAPI (Sigma) was used to stain the nucleus. The number of β-gal (+) cells and BrdU (+) cells were counted using immunofluorescence microscopy.

Cell Transfection and Luciferase Assay

The neuron-specific Tα1 α-tubulin promoter (Gloster *et al.*, 1994) was subcloned into a luciferase reporter vector, pGL2-basic (Promega), to generate the Tα1 α-tubulin promoter-luciferase reported plasmid (Tα1 promoter-Luc). Two Rig-1 expression plasmids were constructed. Rig-1 cDNA encoding either a 1344-amino-acid (amino acid 1–1344) transmembrane form or a 835-amino-acid (amino acid 1–835) secreted form was ligated downstream of a CMV promoter to generate the plasmids Rig-TM or Rig-Sec, respectively. Neuro-2A cells were seeded at a density of 2×10^5 cells/60-mm plate and grown overnight at 37°C. Rig-1 expression construct, either Rig-TM or Rig-Sec, was cotransfected with Tα1 promoter-Luc into Neuro-2A cells using lipofectamine (Gibco BRL, Gaithersburg, MD). The cells were harvested 48 h after transfection and assayed for luciferase activity (Promega).

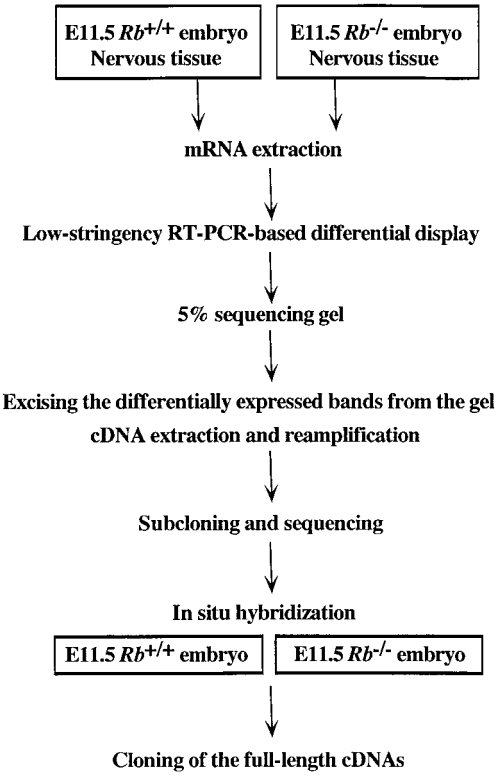


FIG. 1. Scheme for the isolation of nervous tissue-specific abnormally expressed genes in $Rb^{-/-}$ embryos. mRNAs were extracted from specific regions of the embryos for RT-PCR analysis.

RESULTS

Isolation of a Nervous System-Specific cDNA Aberrantly Expressed in $Rb^{-/-}$ Mouse Embryos

To identify genes that were differentially expressed in the nervous system of wild-type and $Rb^{-/-}$ embryos, a low-stringency RT-PCR-based differential display technique (Liang and Pardee, 1992) was used to compare mRNAs extracted from hindbrain, spinal cord, and dorsal root ganglia of E11.5 wild-type ($Rb^{+/+}$) and $Rb^{-/-}$ mouse embryos (Fig. 1). The differentially expressed partial cDNAs were cloned and sequenced. The tissue distribution of one such gene during embryogenesis was studied using *in situ* hybridization (Fig. 2). Full-length cDNA was cloned by screening a mouse E11.5 cDNA library.

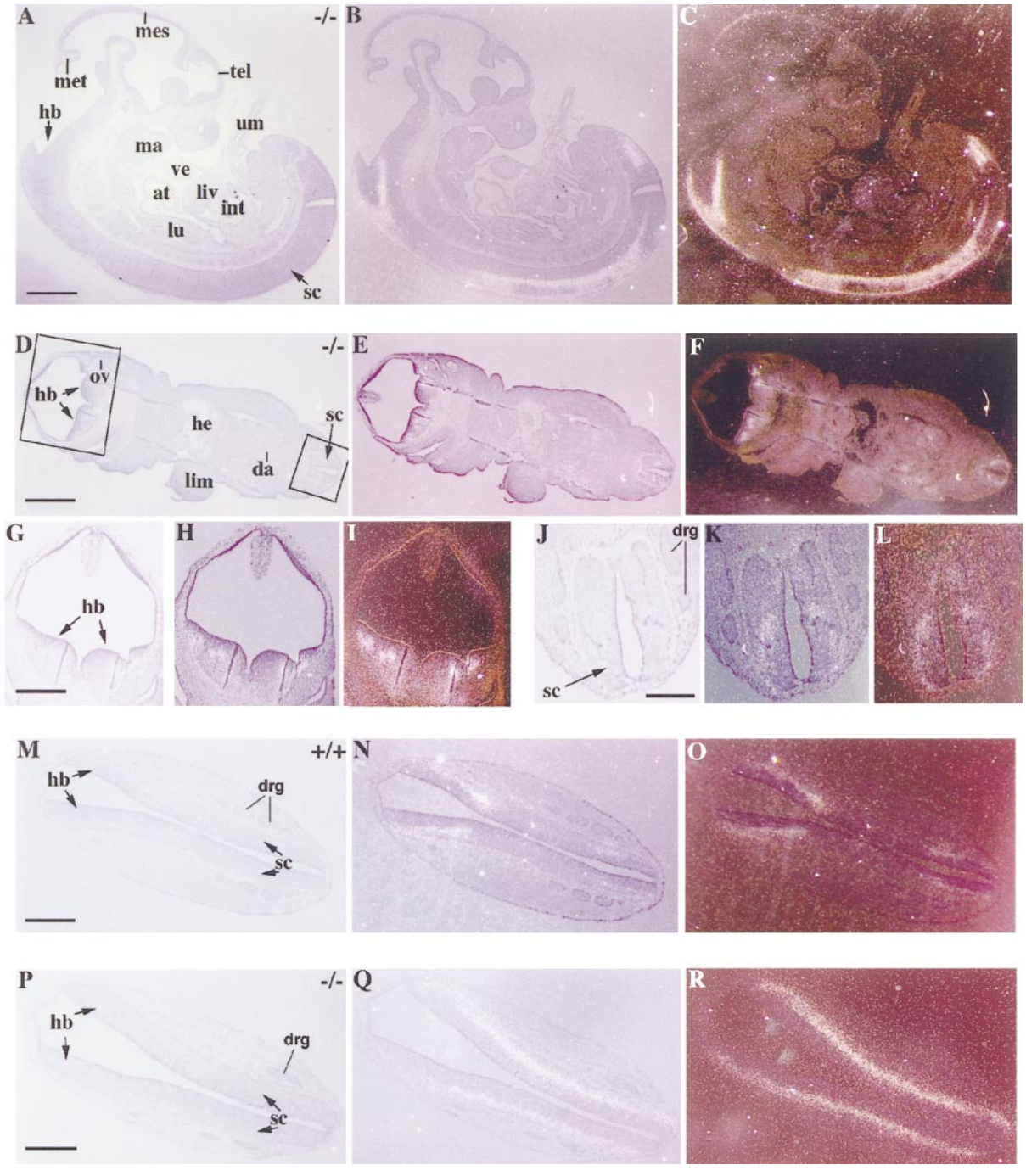


FIG. 2. *In situ* hybridization analysis of Rig-1 transcripts in E11.5 mouse embryos. (A–C) Midsagittal section. (D–R) Coronal section. (A, D, G, J, M, and P) Bright-field views of E11.5 embryo sections. (C, F, I, L, O, and R) Dark-field views of the same sections as in A, D, G, J, M, and P. (B, E, H, K, N, and Q) Superimposition of bright-field views and dark-field views. (+/+) Wild type, (−/−) $Rb^{-/-}$, (at) atrium, (da) dorsal aorta, (drg) dorsal root ganglia, (he) heart, (hb) hindbrain, (int) intestine, (lim) limb, (lu) lung, (ma) mandible, (mes) mesencephalon, (met) metencephalon, (ov) otic vesicle, (sc) spinal cord, (tel) telencephalon, (um) umbilical cord. The two boxed regions in D were magnified and are shown in G–I and J–L, respectively. Scale bar: (A–C) 1000μm, (D–F) 800 μm, (G–I) 240 μm, (J–L) 125 μm, and (M–R) 330 μm.

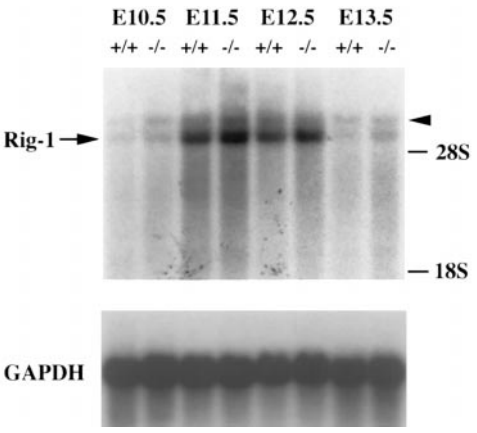


FIG. 3. Northern analysis of Rig-1 transcripts in mouse embryos (Top) Expression of Rig-1 transcripts in various stages of $Rb^{+/+}$ and $Rb^{-/-}$ mouse embryos. (Bottom) GAPDH as internal control. Arrowhead indicates a possible alternatively spliced transcript of Rig-1.

Elevated Expression of a Transiently Expressed Nervous System-Specific Gene, Rig-1, in $Rb^{-/-}$ Mouse Embryos

When antisense oligonucleotides were used as probes for *in situ* hybridization to confirm the differentially expressed cDNAs in $Rb^{+/+}$ and $Rb^{-/-}$ mouse embryos, one of the clones identified by differential display was noticed as being expressed in restricted regions of the embryos, namely the hindbrain and the spinal cord (Fig. 2). These are regions with the most severe neural defect in the $Rb^{-/-}$ embryos (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992, 1994). There was no detectable expression in heart, limb, liver, or lung (Figs. 2C and 2F). The patterns of expression of Rig-1 transcripts were similar between $Rb^{+/+}$ and $Rb^{-/-}$ embryos (Fig. 2O vs 2R). Rig-1 transcripts were expressed in the region between the proliferative cell-containing ventricular zone and the postmitotic cell-containing migration zone (Figs. 2E, 2H, and 2K). Sense oligonucleotides were used as negative controls (data not shown) to demonstrate the specificity of the antisense oligonucleotide probes.

Northern (Fig. 3) and Western (see below) analyses were performed to compare the expression levels of Rig-1 in wild-type and RB mutant embryos. An ~6-kb Rig-1 transcript whose expression was elevated at E11.5 and E12.5 in both $Rb^{+/+}$ and $Rb^{-/-}$ embryos with the peak expression around E11.5 was evident (Fig. 3). There was a moderate increase in the Rig-1 transcript in the $Rb^{-/-}$ embryos (Fig. 3).

The Northern analysis of mouse embryo tissues also showed a faint band, which hybridized with Rig-1 probe (arrowhead in Fig. 3). We speculate that this may be one of the alternatively spliced transcripts of Rig-1 (see Discussion). Rig-1 transcripts were undetectable in 3T3, C2C12, Neuro-2A, P19, PC12, and Rat2 cell lines by Northern analysis (data not shown).

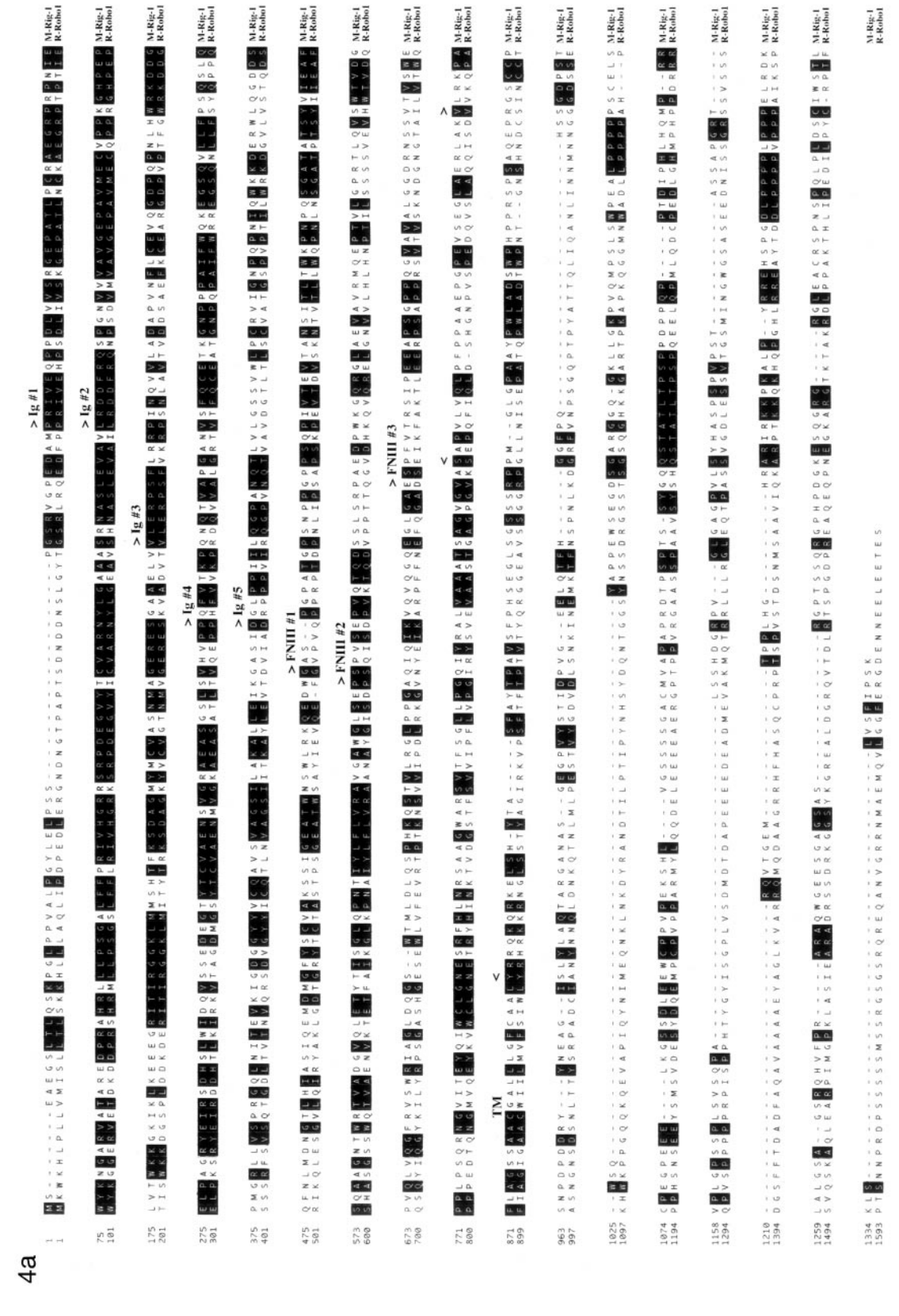
Rig-1 Gene Is a Novel Member of the Immunoglobulin Gene Superfamily

An E11.5 mouse cDNA library (Clontech) was screened using a 350-bp cDNA probe, which was obtained from differential display (Liang and Pardee, 1992). The longest open reading frame, compiled from the overlapping clones, encoded a 1344-amino-acid polypeptide with a N-terminal putative signal peptide, a 846-amino-acid extracellular segment, a 26-amino-acid transmembrane domain, and a 453-amino-acid C-terminal intracellular segment (Fig. 4a). The extracellular segment of Rig-1 contained five immunoglobulin-like (Ig-like) and three fibronectin III-like (FNIII-like) domains, commonly present in growth factor receptors and proteins involved in cell adhesion and differentiation (reviewed by Reichardt and Tomaselli, 1991; Holness and Simmons, 1994). Rig-1 shared 40% amino acid identity with rat and human membrane protein roundabout (Robo1), which plays a role in axon crossing of the CNS midline (Seeger *et al.*, 1993; Kidd *et al.*, 1998) (Fig. 4a). Rat roundabout (R-Robo1) protein also contains five Ig-like and three FNIII-like domains in its extracellular segment. Rig-1 and R-Robo1 share 53% overall amino acid identity in the extracellular segment, 48% identity in the transmembrane domain, and 26% identity in the intracellular segment (Fig. 4b). Other than the cytoplasmic motif 3, a characteristic of roundabout family proteins, the remaining intracellular segment of Rig-1 does not share high homology with known proteins (Fig. 4b).

During the cloning of Rig-1 cDNA, various alternatively spliced exons were noticed (Fig. 4c). At least nine alternatively spliced exons were identified. Stop codons were found in the alternatively spliced exons 1, 2, 3, 5, 6, 7, and 9, but not in exon 4 or 8. Therefore, Rig-1 cDNAs are predicted to encode either a transmembrane form or a secreted form of the protein.

Identification of Rig-1 Protein in the Central Nervous System of Mouse Embryos

To characterize Rig-1 protein product, rabbit antiserum against amino acids 376–929 (anti-ECD) and mouse antiserum against the intracellular domain (amino acids 1067–1234) (anti-ICD) were produced (Fig. 5a). Both anti-ECD and anti-ICD antisera reacted with an ~210-kDa protein band in a Western blot of cell lysates extracted from the hindbrain and spinal cord of E11.5 $Rb^{-/-}$ mouse embryos (Fig. 5b, lanes 1 and 5). The ~210-kDa protein band was also evident when the antisera were used to immunoprecipitate the protein followed by Western blot using the same (Fig. 5b, lanes 3 and 7) or the other (lane 9) antibody. Preimmune sera did not react with the ~210-kDa protein band in immunoprecipitation (Fig. 5b, lane 2 and 6) or in Western analysis (data not shown). Additionally, use of excess pro-



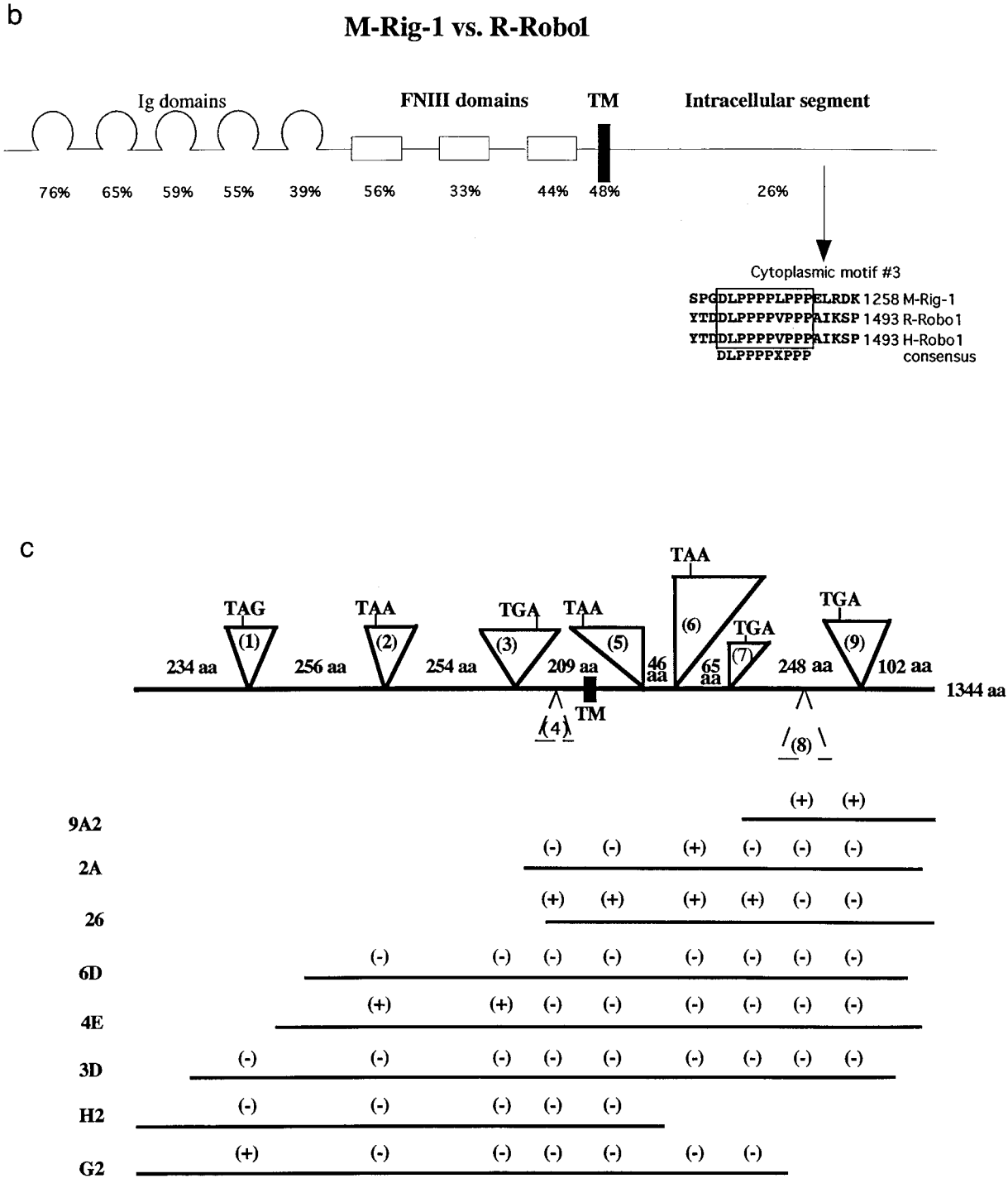


FIG. 4. Sequence comparison of mouse Rig-1 with roundabout proteins. (a) Amino acid sequence alignment using MegAlign program (DNASTAR, Madison, WI). Mouse Rig-1 (M-Rig-1) was compared with rat roundabout (R-Robol; GenBank Accession No. AF041082). (Ig) immunoglobulin-like domain, (FNIII) fibronectin III-like domain, (TM) transmembrane domain. (b) Domain structure analysis of M-Rig-1 and R-Robol. Percentages shown indicate the amino acid identity between M-Rig-1 and R-Robol of each domain. (c) Alternative splicing of Rig-1. The alternatively spliced exons are labeled from 1 to 9 in parentheses. Two of the alternatively spliced exons are in frame (4 and 8) and the others contain stop codons. Eight cDNA clones are listed to show the presence (+) or absence (−) of each alternatively spliced exon.

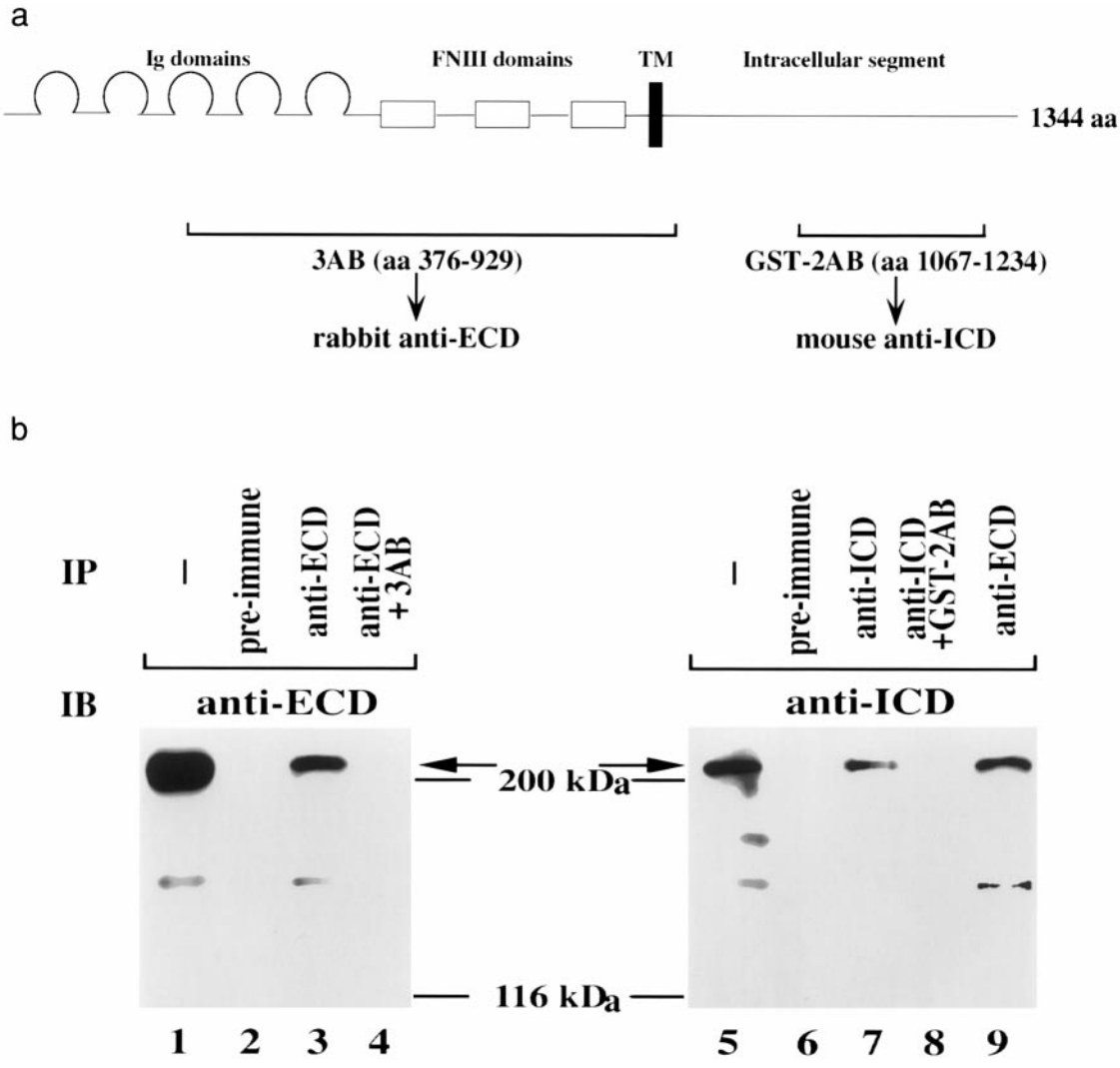


FIG. 5. Identification of Rig-1 protein in the developing mouse central nervous system. (a) Schematic drawing showing the regions of Rig-1 protein (named 3AB and GST-2AB) used as antigens to generate rabbit anti-ECD (against amino acids 376–929) and mouse anti-ICD (against amino acids 1067–1234). (b) (Left) Western blot analysis of total cell lysates extracted from hindbrain and spinal cord of E11.5 $Rb^{-/-}$ mouse embryos using rabbit anti-ECD (lane 1); alternatively, immunoprecipitation was performed using rabbit preimmune IgG (lane 2) or rabbit anti-ECD prior to Western blot analysis (lanes 3 and 4). In lane 4, 3AB was added during immunoprecipiation. (Right) Western blot analysis of total cell lysates using mouse anti-ICD (lane 5); alternatively, immunoprecipitation was performed using either mouse preimmune IgG (lane 6) or mouse anti-ICD (lanes 7 and 8) prior to the Western blot analysis. In lane 8, GST-2AB was added during immunoprecipitation. In lane 9, rabbit anti-ECD and mouse anti-ICD were used for immunoprecipitation and Western analysis sequentially. (IP) immunoprecipitation, (IB) immunoblotting.

tein antigens 3AB or GST-2AB in immunoprecipitation resulted in an absence of the ~210-kDa band (Fig. 5b, lens 4 and 8), suggesting that it was indeed the endogenous Rig-1 protein. An ~160-kDa protein band whose identity is unknown was always present in immunoblots using rabbit anti-ECD antisera or mouse anti-ICD antisera in various mouse tissues (Figs. 5b and 7).

Rig-1 Is a Membrane-Associated Protein

Protein sequence analysis suggested that Rig-1 is likely a transmembrane protein. Since Rig-1 protein was expressed in the hindbrain and spinal cord of E11.5 $Rb^{-/-}$ mouse embryos at high levels, mouse primary cells from hindbrain and spinal cord of E11.5 $Rb^{-/-}$ embryos were fractionated.

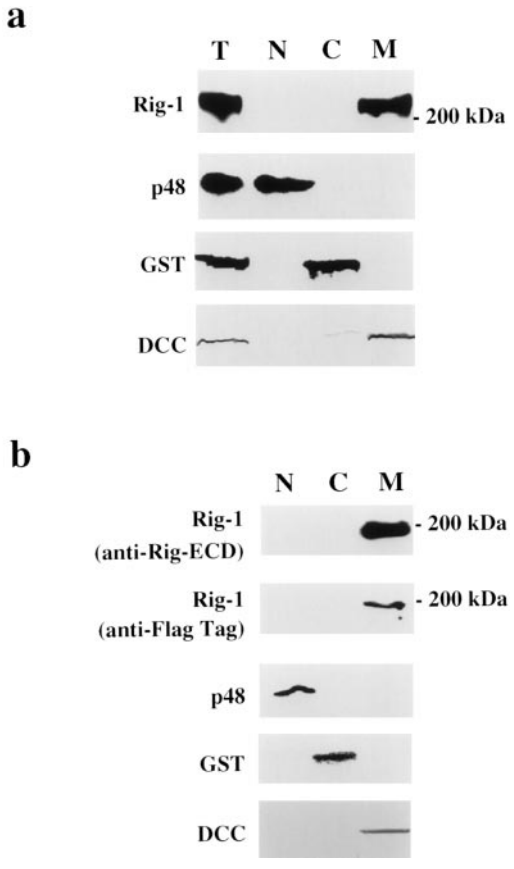


FIG. 6. Rig-1 protein distribution in different cellular fractions. (a) Subcellular localization of endogenous Rig-1 protein. Primary cells from hindbrain and spinal cord of E11.5 mouse embryos were fractionated into nuclear, cytoplasmic, and membranous fractions. Proteins p48 (an RB-associated protein), GST (glutathione-*S*-transferase), and DCC (deleted in colorectal cancer) were used as indicators for nuclear, cytoplasmic, and membranous fractions, respectively. (b) Subcellular localization of Flag-tagged Rig-1. Neuro-2A cells were transfected with pCEP4F-Rig, which encoded Flag-tagged Rig-1 (amino acids 1–1344), and subjected to subcellular fractionation. (T) total cell lysate, (N) nuclear fraction, (C) cytoplasmic fraction, (M) membranous fraction.

Rabbit anti-ECD antiserum reacted with an ~210-kDa protein only in the membranous fraction, indicating that Rig-1 was localized to the membrane (Fig. 6a). p48 (an RB-associated protein), GST (glutathione-S-transferase), and DCC proteins were used as markers of nuclear, cytoplasmic, and membranous fractions, respectively. Additionally, Neuro-2A cells were transfected with the Flag-tagged Rig-1 cDNA (pCEP4F-Rig) which encodes a 1344-amino-acid Rig-1 protein containing a putative transmembrane domain. Rabbit anti-ECD antiserum as well as anti-Flag monoclonal antibody reacted with an ~195-kDa protein band only in the membranous fraction and not in nuclear or cytoplasmic fractions in a Western blotting analysis of transfected cell lysates (Fig. 6b), confirming that Flag-tagged Rig-1 was also a transmembrane protein. Therefore, both endogenous and recombinant Rig-1 proteins are transmembrane proteins.

Expression of the Rig-1 Protein Is Nervous System-Specific and Elevated in the $Rb^{-/-}$ Embryos

Since the level of Rig-1 mRNA expression was higher in the $Rb^{-/-}$ embryos compared to the $Rb^{+/+}$ embryos (Figs. 2 and 3), we tested the pattern and expression level of Rig-1 protein in mouse embryos. Western blot analysis suggested that Rig-1 protein expression was nervous system-specific and transient during embryonic development (Fig. 7 and data not shown). Consistent with the Rig-1 mRNA expression, Rig-1 protein was expressed in the hindbrain and spinal cord of $Rb^{+/+}$ and $Rb^{-/-}$ mouse embryos with the peak expression around E11.5. Additionally, an elevated level of the ~210-kDa Rig-1 protein was detected in the hindbrain–spinal cord of $Rb^{-/-}$ embryos and no ~210-kDa Rig-1 protein was detected in the liver of mouse embryos, brain of newborn mice, or brain of adult mice. RB-associated protein p48 was used as an internal control; it is expressed at a higher level in embryonic tissues compared to those of newborn or adult mice. However, no variation in the level of p48 expression was detected between $Rb^{+/+}$ and $Rb^{-/-}$ mouse tissues (Fig. 7, bottom). Rig-1 protein was also undetectable in the forebrain, heart, kidney, limb, lung, and spleen tissues of mouse embryos nor in 3T3, C2C12, Neuro-2A, P19, PC12, and Rat2 cell lines (data not shown) when 200 μg total cell lysate was used for Western blot analysis, indicating the highly temporal and tissue-restricted expression pattern of Rig-1.

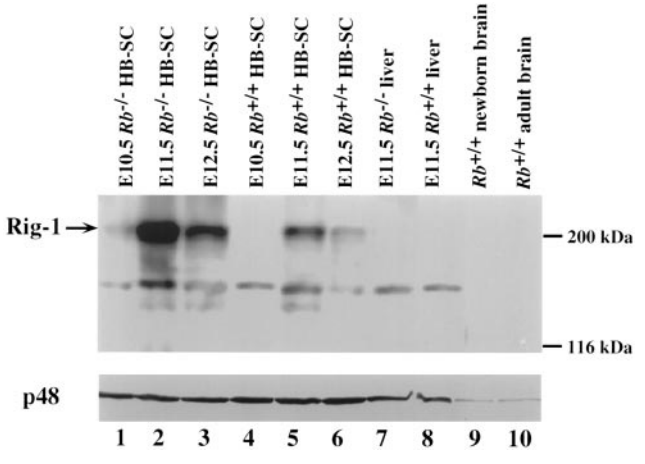


FIG. 7. Expression of ~210-kDa Rig-1 protein during embryogenesis in $Rb^{+/+}$ and $Rb^{-/-}$ mouse embryos. (HB-SC) Hindbrain and spinal cord. 200 μg of total cell lysate from different tissues was subjected to Western blotting analysis using rabbit anti-ECD antibody. RB-associated protein p48 was used as an internal control.

Ectopic Expression of the Transmembrane Form of Rig-1 Protein, But Not the Secreted Form, Enhanced Cell Entrance into S Phase

The lack of terminal differentiation, failure to exit cell cycle, and extensive apoptosis have been reported for the RB-deficient neurons (Lee *et al.*, 1994). To test whether overexpression of Rig-1 protein may mediate some of these defects, Rig-1 expression vectors were introduced into neuronal cell line. Neuro-2A cells were cotransfected with CS+nucβ-gal (Fine *et al.*, 1990) and Rig-1 expression constructs of transmembrane form or secreted form (amino acid 1–1344 transmembrane form, Rig-TM, or amino acid 1–835 secreted form, Rig-Sec) and labeled with BrdU followed by anti-BrdU and anti-β-galactosidase antibodies. Thus red fluorescence and green fluorescence represented cells that were positive for BrdU and β-galactosidase staining, respectively (Fig. 8a). Expression of the transmembrane form of Rig-1 corresponded with 46% of the cells in S phase, while only 28 and 23% of the cells were in S phase when transfected with the control vector or the secreted form of Rig-1, respectively (Fig. 8b). RB and E2F-1 expression constructs were included to serve as controls in the transfection for inhibition and promotion of cell proliferation, respectively. The enhancement of cell proliferation by the transmembrane form of Rig-1 protein was similar to that of E2F in this assay (Fig. 8b).

Ectopic Expression of the Transmembrane Form of Rig-1 Protein, But Not the Secreted Form, Repressed the Differentiated Neuron-Specific Tα1 α-Tubulin Promoter

To test the effect of Rig-1 on neuron differentiation, a reporter plasmid consisting of a differentiated neuron-specific Tα1 α-tubulin promoter (Gloster *et al.*, 1994) and luciferase cDNA (pGL2-basic) was constructed. Coexpression of the 1344-residue transmembrane form of Rig-1, but not the 835-amino-acid secreted form of Rig-1, and the Tα1 α-tubulin promoter–luciferase reporter construct in Neuro-2A cells caused a 2.5-fold decrease in the promoter activity (Fig. 9) in a dose-dependent manner. Transmembrane and secreted forms of Rig-1 had no effect on expression of luciferase activity of the control vector (data not shown).

DISCUSSION

Analysis of mouse embryos lacking RB protein demonstrated its pleiotropic function in multiple cell types. Rig-1 is a novel gene, identified as one of the potential downstream effector genes of RB. Here we characterized the Rig-1 expression pattern and the protein product. In addition, preliminary functional studies suggested that some of the neuronal defects seen in the $Rb^{-/-}$ mouse embryos may be mediated by the deregulation of Rig-1 expression.

Rig-1 Is a Novel Nervous System-Specific Gene That Is Aberrantly Expressed in $Rb^{-/-}$ Embryos

Northern analysis (Fig. 3) and Western blotting (Fig. 7) indicate that Rig-1 mRNA and protein levels are increased in RB-mutant embryos. Aberrant expression of both ubiquitous and tissue-specific downstream effectors of RB is well documented. While examples like E2F and cyclin E belong to the former group (Herrera *et al.*, 1996; Yamasaki *et al.*, 1998), MyoD, trkA, low-affinity NGF receptor p75, and βII tubulin (Gu *et al.*, 1993; Lee *et al.*, 1994) are examples of the latter. Since the E2F binding site is present in the cyclin E promoter and since derepression of E2F transcriptional activity occurs in the absence of RB, the enhanced expression of cyclin E is likely due to deregulation of E2F activity (Ohtani *et al.*, 1995). On the other hand, mechanisms leading to aberrant expression of tissue-specific genes in the absence of RB are largely unknown.

Two potential mechanisms may underlie the aberrant expression of Rig-1. Cell populations expressing Rig-1 may be enriched due to the loss of non-Rig-1-expressing cells in the developing nervous system. Alternatively, Rig-1 expression may be regulated directly or indirectly by RB. While additional studies will be required to clarify the cause of increased expression of Rig-1 in the nervous system of the RB-mutant embryos, our preliminary data suggest it may be partially due to the derepression of the Rig-1 promoter in the absence of RB (S.-S. F. Yuan *et al.*, unpublished data). In view of the restricted spatial and temporal expression of Rig-1, its promoter is likely to be subject to regulation by neuron-specific transcriptional factors. Future studies are needed to understand the regulation of Rig-1 promoter activity.

Alternatively Spliced Forms of Rig-1

The longest open reading frame of the Rig-1 cDNA that was cloned encoded a protein with 1344 amino acids and a predicted molecular mass of 180 kDa. When expressed in mammalian cells, the protein migrated with an apparent molecular mass of ~195 kDa under denaturing conditions (Fig. 6b). The discrepancy between the predicted and the observed molecular mass may be due to glycosylation since treatment with glycosidases, neuraminidase or Endo F, resulted in a faster migrating protein product (S.-S. F. Yuan *et al.*, unpublished data). In mouse embryos, an ~210-kDa protein band was specifically recognized by both anti-ECD and anti-ICD antibodies, indicating that the endogenous full-length Rig-1 protein product is about 210 kDa in size. One explanation for the difference in size seen between the recombinant and the endogenous Rig-1 protein may be that our "longest open reading frame" of the Rig-1 cDNA may lack some in-frame alternatively spliced exon(s). Although 19 different Rig-1 cDNAs were cloned, none of them contained a longer open reading frame that would account for the ~210-kDa protein product. Another possibility is that glycosylation of Rig-1 in embryos may differ from that in cultured cells and thus result in a more slowly migrating

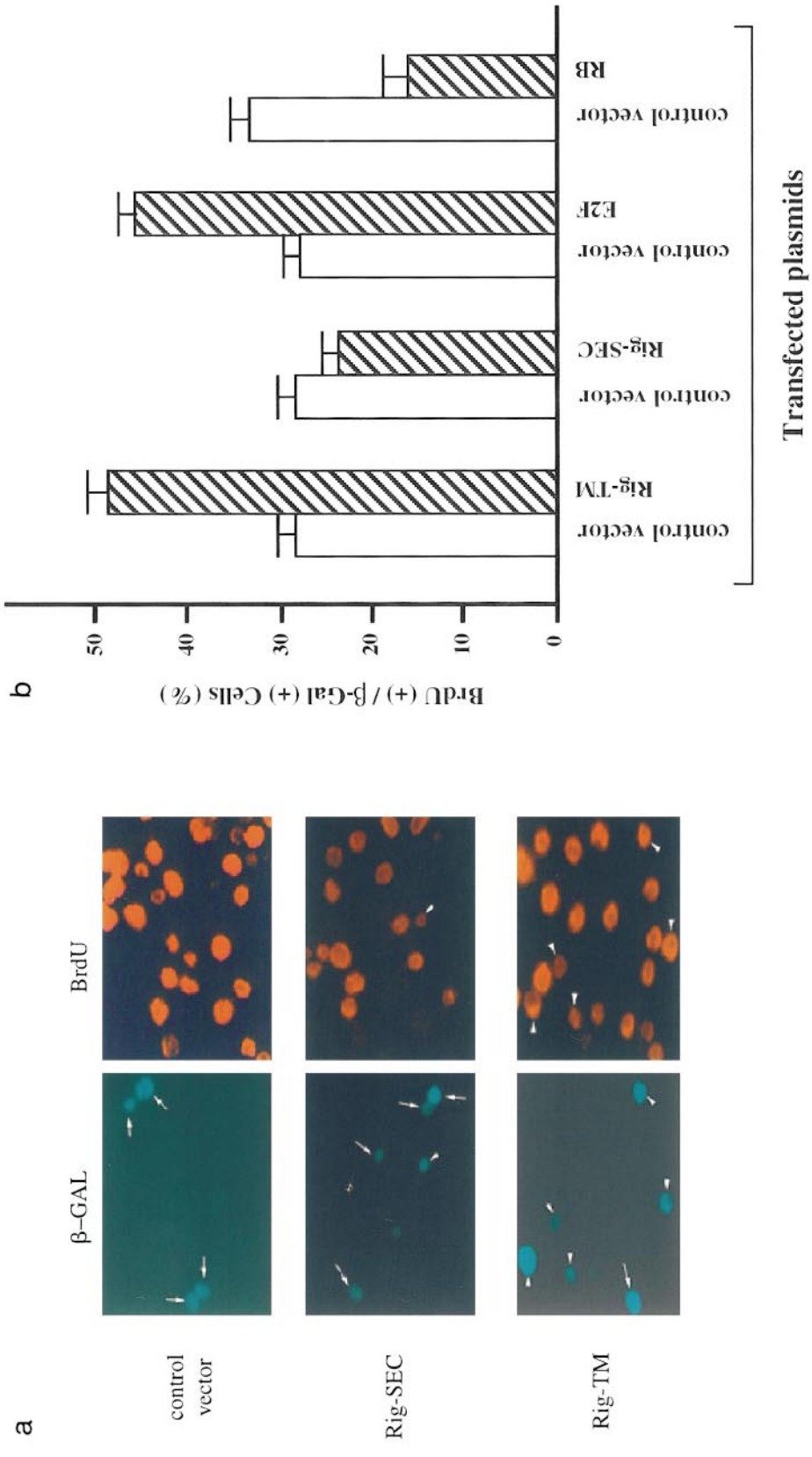


FIG. 8. Effects of the transmembrane form (amino acids 1–1344) and secreted form (amino acids 1–835) of Rig-1 protein on S-phase progression. (a) FITC-conjugated secondary antibody was used to detect β-galactosidase-positive cells and rodamine-conjugated secondary antibody was used to detect BrdU-positive cells. (Arrow) Cells positive for β-galactosidase staining but negative for BrdU staining, (arrowhead) cells double positive for both β-galactosidase and BrdU, (Rig-TM) transmembrane form of Rig-1 protein, (Rig-SEC) secreted form of Rig-1 protein. (b) Histogram of percentage of double-positive cells in the β-galactosidase-positive cells. More than 1000 double-positive cells were counted. The percentages of double-positive cells are presented as mean ± SEM based on three sets of experiments.

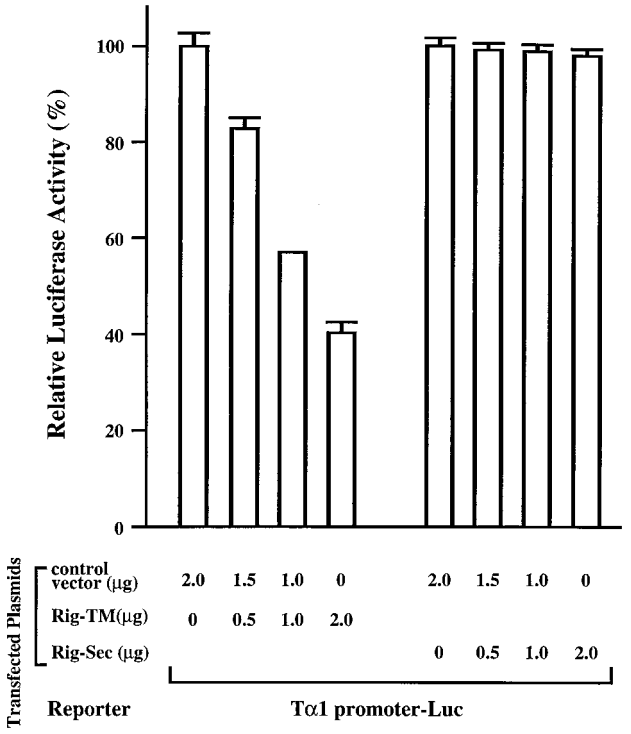


FIG. 9. Effects of the transmembrane form and secreted form of Rig-1 protein on neuron-specific Tα1 α-tubulin promoter activity. Individual Rig-1 expression constructs, Tα1 α-tubulin promoter–luciferase reporter construct (Tα1 promoter-Luc), and CS+nucβ-gal were contransfected into Neuro-2A cells. Luciferase activities were assayed 48 h after transfection and normalized using β-galactosidase activity as the internal control. The result are presented as mean ± SEM based on four sets of experiments. (Vector) pCMV vector for subcloning of different Rig-1 cDNA fragments, (Rig-TM) transmembrane form of Rig-1 protein (amino acids 1–1344), (Rig-Sec) secreted form of Rig-1 protein (amino acids 1–835).

protein product. Additionally, an ~160-kDa band cross-reacted with rabbit anti-ECD antisera and mouse anti-ICD antisera in various mouse tissues (Figs. 5b and 7), which we speculate may be a cross-reacting protein or an alternatively spliced form of Rig-1 protein. Determination of which of the two possibilities is true awaits further experimentation.

More than nine differentially spliced Rig-1 exons were discovered (Fig. 4c). Some of the spliced forms contained stop codons in the alternatively spliced exons and therefore encoded a secreted protein rather than a transmembrane protein. Examples of differential splicing in neurons abound and alternative transcripts may encode proteins with specific functions (Hortsch *et al.*, 1990; Reale *et al.*, 1994; Pierceall *et al.*, 1994). Different forms of Rig-1 protein are likely to have unique cellular functions. In support of this hypothesis we observed that overexpression of the transmembrane form of Rig-1 protein, but not the secreted form, enhanced S-phase progression, and repressed the neuron-specific Tα1 α-tubulin promoter (Figs. 8 and 9). Western blot analysis demonstrated that expression of the ~210-kDa transmembrane form of Rig-1 protein was elevated in $Rb^{-/-}$ embryos. Whether expression of secreted form of Rig-1 is also affected in a similar fashion requires further studies.

Rig-1 May in Part Mediate Neuronal Defects Seen in $Rb^{-/-}$ Mouse Embryos

The effect of Rig-1 overexpression on neuronal proliferation and differentiation was studied in the mouse neuronal cell line, Neuro-2A. Endogenous Rig-1 was undetectable in Neuro-2A cells (data not shown). While transmembrane proteins such as roundabout (Seeger *et al.*, 1993; Kidd *et al.*, 1998; Zallen *et al.*, 1998) and DCC (Keino-Masu *et al.*, 1996; Fazeli *et al.*, 1997) are important for neuronal development, signal events through their intracellular domain are unclear. Ectopic expression of the transmembrane but not the secreted form of Rig-1 in Neuro-2A cells enhanced S-phase entry (Fig. 8), indicating that the intracellular domain is crucial for Rig-1 activity. While studies of overexpression of Rig-1 in Neuro-2A cells suggest a role in cell proliferation, further studies such as a transgenic approach may help address if overexpression of Rig-1 has mitogenic effect on neurons.

The decreased activity of Tα1 α-tubulin promoter in Neuro 2A cells overexpressing Rig-1 (Fig. 9) could be a consequence of the mitogenic effect of Rig-1 which in turn inhibits differentiation. The activity of this neuron-specific promoter appears to be normal in RB-mutant embryos at E12.5 (Slack *et al.*, 1998). In addition, the temporal expression pattern of Rig-1, being the highest at E11.5 and diminishing by E13.5, does not support an inhibitory role of Rig-1 on Tα1 α-tubulin promoter.

Although both the central nervous system (CNS) and the peripheral nervous system (PNS) are affected in the $Rb^{-/-}$ embryos, Rig-1 is expressed only in limited areas of the CNS. Therefore, we speculate that other genes may mediate neuronal defects seen in the PNS and in areas other than hindbrain and spinal cord in the CNS of $Rb^{-/-}$ embryos. Whether any members of the roundabout gene family are expressed in the PNS awaits further studies.

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